Part 9 #12
8.20.96

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	**Michael Meyrick Burrell and Keith Stuart Blundy**
Serial No.:	**08/284,199**
Filed:	**August 2, 1995**
Art Unit:	**1804**
For:	**MODIFICATION OF PLANT METABOLISM**
Examiner:	**D. Fox**

DECLARATION UNDER RULE 37 CFR 1.132

I, **Michael Meyrick BURRELL**, of 20 Histon Road, Cottenham, Cambridge, England, **DO SOLEMNLY** and **SINCERELY DECLARE** as follows:

1. I am the **Michael Meyrick Burrell** who is named as being an Applicant in respect of United States Patent Application Serial No. 08/284,199.

2. I am a scholar of Cambridge University and hold a PhD in Plant Biochemistry and a Degree in Natural Sciences with honours in Botany.

3. I initiated and supervised the conduct of the experimental procedures now to be related.

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4. Potato plants were transformed with a chimaeric gene comprising a patatin promoter, an antisense **invertase** coding sequence and a nos terminator. The bar chart attached as Exhibit A shows results, in terms of the Y-axis, of the ratio sucrose to glucose + fructose. (On the chart "sem"=standard error of the mean).

5. The bar chart clearly shows that plants were obtained with a greatly increased ratio of sucrose to glucose + fructose. This is a commercially advantageous attribute in certain forms of potato processing. The person skilled in the art could readily select such plants and clonely multiply them, so to obtain commercial quantities thereof.

6. Nine independent transformed lines were grown in a paired experiment with each line replicated ten times. As is shown by the results table below, the reducing sugar content, as represented by the content of glucose, is significantly less in the transgenic plants than in control plants, whereas in the transgenic plants the sucrose:glucose ratio is significantly increased relative to the control plants.

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	Glucose mg/g fr wt			Ratio Sucrose:Glucose	
Line	Transgenic	Control	P	Transgenic	Control
2273	0.626	1.238	0.002	2.57	0.73
2062	0.363	0.749	0.006	4.26	2.19
2134	0.739	1.505	0.01	2.15	0.62
2263	0.923	1.299	0.015	2.24	1.22
2295	1.208	2.095	0.015	1.28	0.71
2042	1.499	2.054	0.024	0.98	0.56
2016	0.832	1.248	0.034	1.35	0.83
2294	0.996	1.418	0.035	1.15	0.67
2028	1.118	1.394	0.047	1.00	0.89

[In the results Table, P is Fischers Probability. Statistical difference is generally accepted if P is less than 0.05].

7. In a second experimental procedure potatoes transformed with the sequence for **sucrose synthase** in a chimaeric gene which also comprised a patatin promoter and a nos terminator. A field trial of a randomised block design was conducted, in which trial there were three replicates. An analysis of variance in regard to the specific gravity (an indicator of starch content) for the three lines indicates that they were significantly different from controls (standard error of difference = 0.0075).

Line	Specific Gravity
89	1.168
36	1.145
52	1.137
Control	1.117

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8. In a third experimental procedure the coding sequence for the **wheat homologue of *waxy*** was fused to the high molecular weight glutenin promoter of wheat to produce a chimaeric gene which would be expressed in developing endosperm. A hybrid maize was transformed with this construct using the particle bombardment method and plants were regenerated to produce seed by outcrossing.

9. Analysis of single seeds taken from six independent transformants is shown below. Starch was isolated and the starch granule bound proteins separated by gel electrophoresis. The 60kDa waxy protein is clearly absent from line 3 (Figure 1). The starch from this line clearly has a much reduced amylose content (Figure 2).

Fig.1.

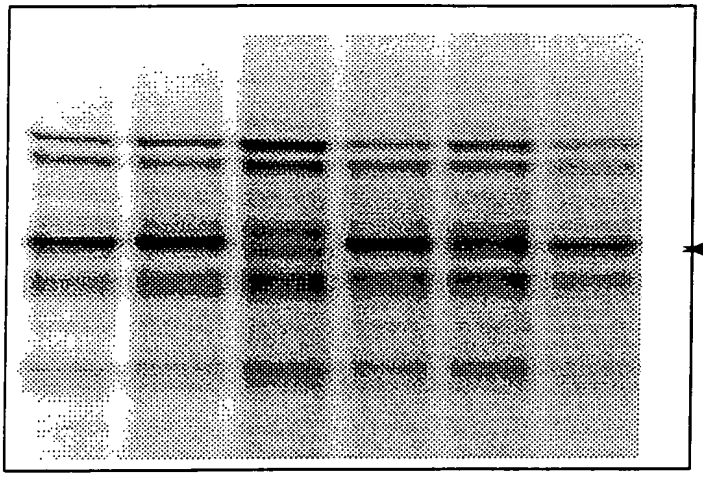
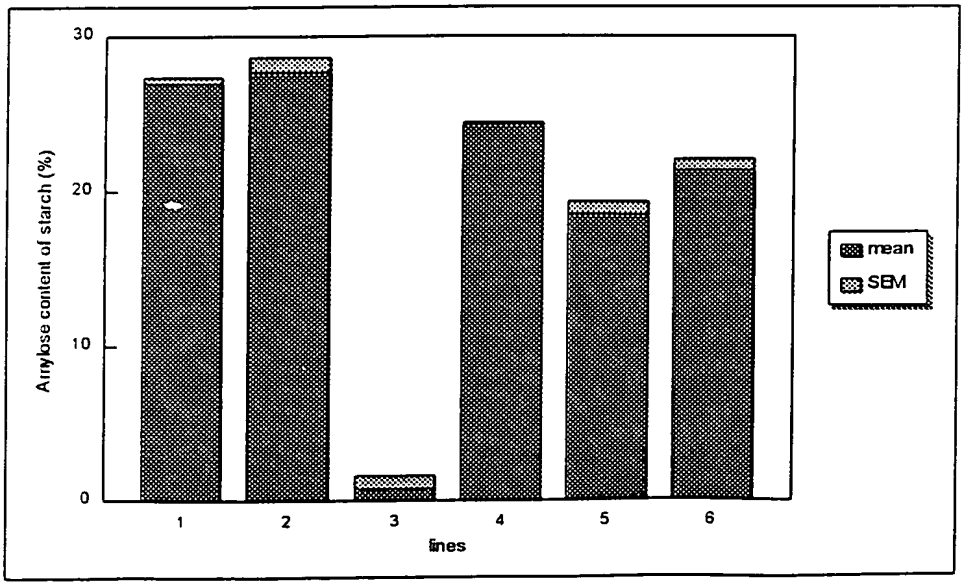


Fig.2.



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10. The seed from the progeny of plants obtained in the transformation procedure were divided into two populations. One population contained plants that showed no clear 60kDa waxy protein product and one population contained segregants that had the 60kDa waxy protein. A third population of untransformed controls were also included in the analysis. The results (table below) clearly show the somewhat surprising result that the absence of the waxy protein is associated with no detectable amylose and no change in starch content.

	Starch Content umol/gfwt		% amylose		
Grain analysed	**Mean**	**S.E.M.**	**Mean**	**S.E.M.**	**Number of grain analysed**
Control line grain	91.30593	13.83537	19.28946	1.834417	5
Progeny grain lacking the product of the waxy gene	105.9155	11.64986	nd*	nd*	15
Progeny grain expressing the product of the waxy gene	92.26848	8.171925	21.92222	0.71156	16

***nd = not detected**

11. In a fourth experimental procedure the full length antisense sequence for **sucrose phosphate synthase** (SPS) from spinach was substituted for the PFK sequence in the vector described in SN 08/284,199. The chimaeric gene was then introduced into potato by transformation with Agrobacterium and potato plants regenerated.

12. To examine the effect of this chimaeric gene on sugar content, five control plants and five clonal plants of the same transgenic line were grown adjacent to each other in soil in a greenhouse.

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This was to ensure as far as was possible that the water supply and light environment of the control and transgenic plants were the same.

13. Mature tubers were harvested from the potato plants and after killing in boiling 80% ethanol the sugars were recovered by aqueous extraction. Sucrose and glucose were then determined enzymatically as described by Morrell, S. and ap Rees, T. (1986) Phytochemistry 25: 1579-1585.

14. The results are presented in the table below and in graphical form (Exhibit B). As is usual, there is considerable variation in sugar content between different tubers. Each transgenic line is therefore compared with its paired non-transgenic control and to aid comparison the percentage change for each line with respect to its paired control has been calculated. In the table the results have been ranked by this percentage change for glucose.

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Line #	P Value	Glucose (mg/g)		Percent	P Value	Sucrose (mg/g)		Percent
		Control	Transgenic	Change		Control	Transgenic	Change
9657	0.036	0.717	0.545	24	0.023	1.686	2.015	120
9618	0.018	1.171	0.772	34	0.021	1.813	2.254	124
9686	0.002	0.979	0.486	50	0.703	1.463	1.503	103
9628	0.048	0.805	0.374	54	0.017	1.68	1.397	83
9630	0.006	0.519	0.227	56	0.475	1.198	1.25	104
9585	0.011	1.297	0.56	57	0.25	1.63	1.457	89
9660	0.017	0.57	0.223	61	0.503	1.029	1.007	98
9588	0.017	0.571	0.198	65	0.556	1.462	1.359	93
9591	0.036	0.536	0.186	65	0.006	1.458	1.728	119
9619	0.036	1.233	0.416	66	0.41	1.427	1.497	105
9661	0.011	0.747	0.249	67	0.373	1.313	1.269	97
9614	0.009	0.685	0.217	68	0.535	1.587	1.623	102
9592	0.018	0.33	0.076	77	0.139	1.474	1.159	79
9570	0.001	0.602	0.114	81	0.016	1.329	1.908	144
9549	0.007	0.471	0.074	84	0.863	1.807	1.782	99
9669	0.001	0.615	0.09	85	0.048	1.662	1.892	114
9558	0.016	0.88	0.112	87	0.245	1.689	1.971	117
9554	0.015	0.416	0.039	91	0.924	1.762	1.755	100
9651	0.001	0.598	0.046	92	0.018	1.762	2.231	127
9605	0.023	0.591	0.024	96	0.152	1.723	2.082	121

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15. It is notable that while SPS is the enzyme which synthesises sucrose, the greatest impact of the chimaeric gene is on the amount of glucose (a reducing sugar).

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of the application or any patent issuing thereon.

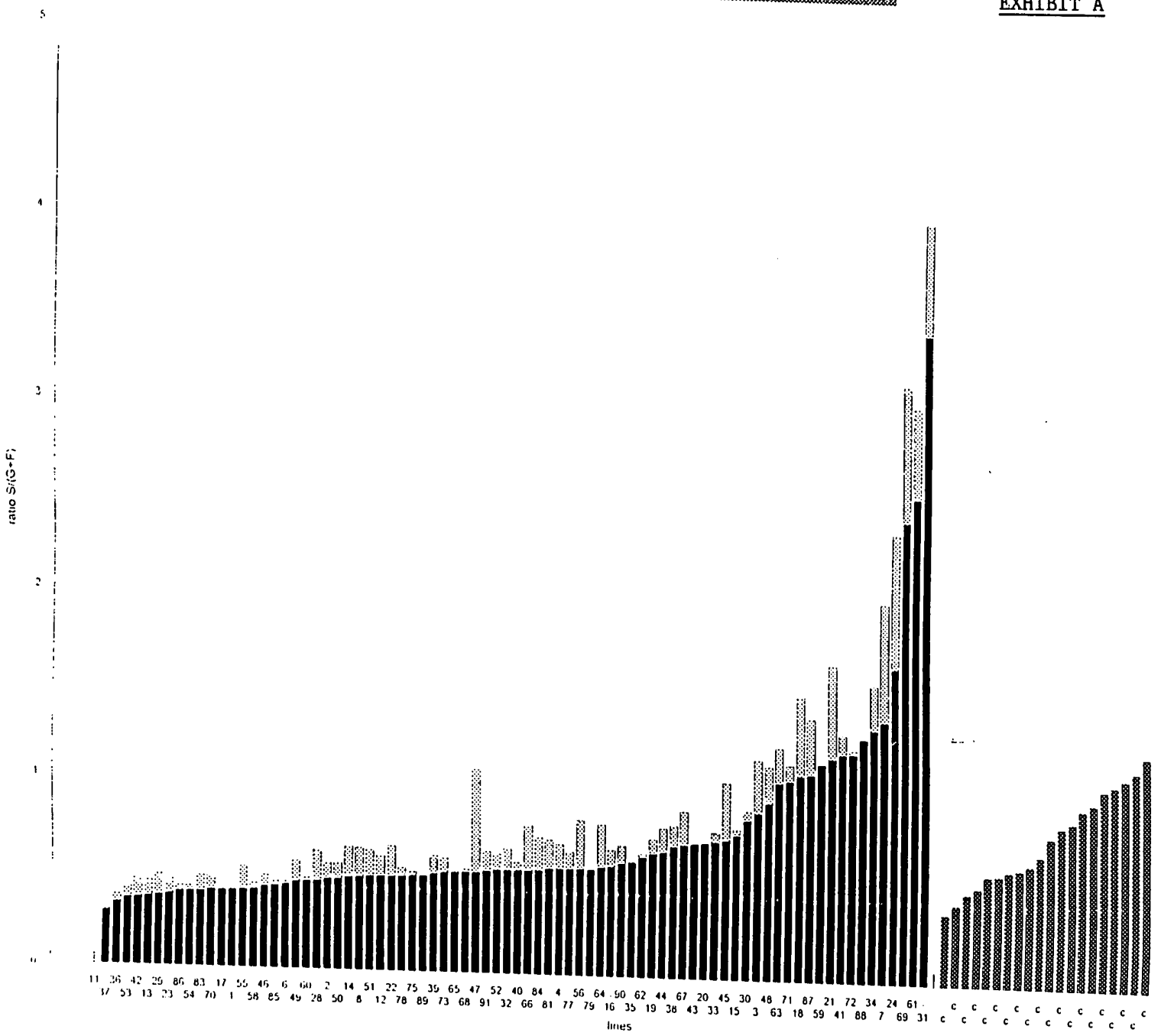
Signature: Michael M. Bevell

Date: 22 June 1996

Ratio of sucrose to reducing sugars in potato tubers.

Greenhouse grown plants

EXHIBIT A



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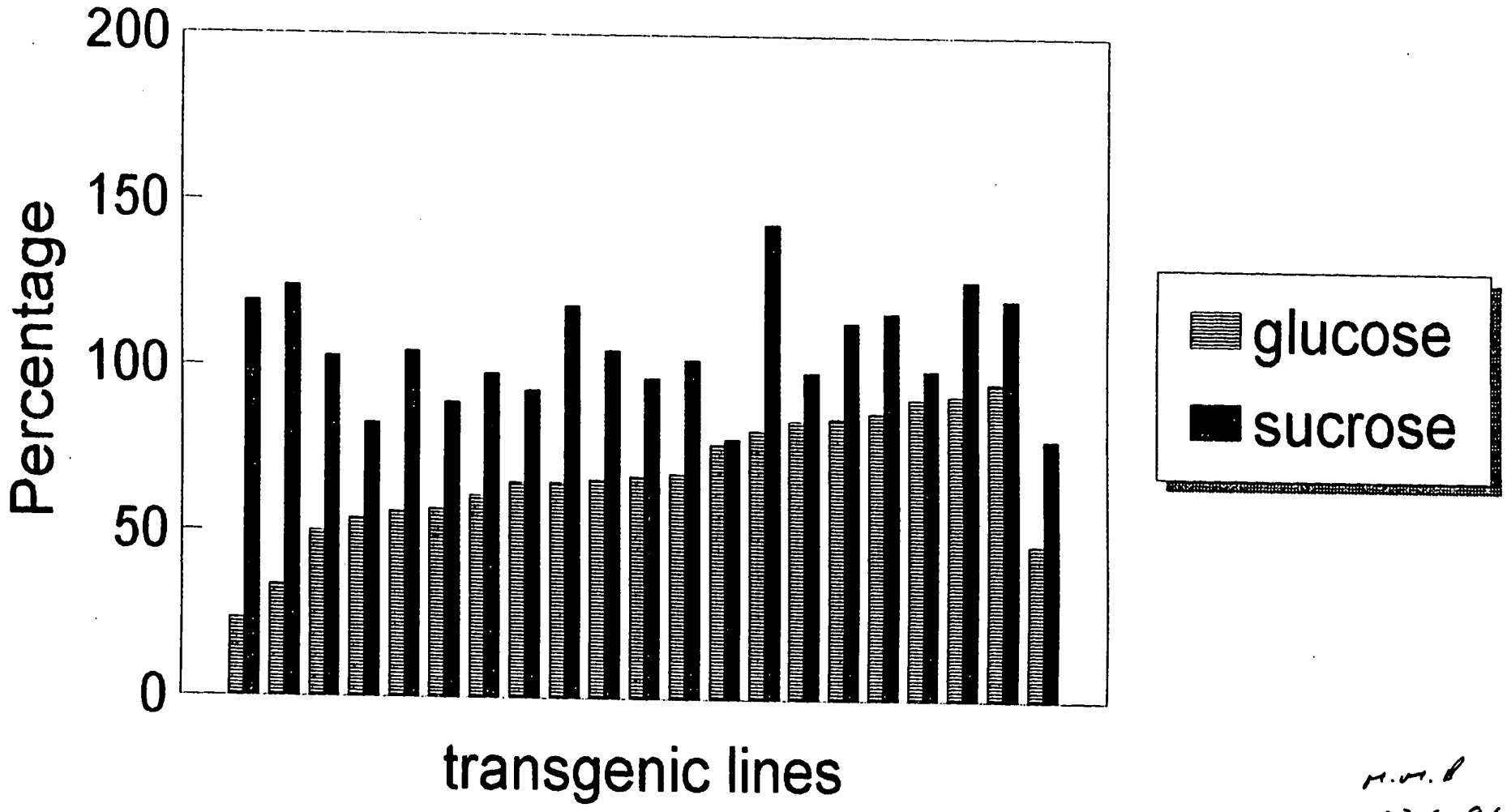
Percentage change in transgenic line compared with paired control



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